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Taxonomic re-evaluation and phylogenetic position of *Hemibeltrania cinnamomi* within *Xylariales*

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Abstract — *Hemibeltrania cinnamomi* associated with leaf blotch disease of *Cinnamomum malabatrum* (*Lauraceae*) is described and illustrated. Phylogenetic LSU and ITS sequence analyses support the fungus as a species of *Hemibeltrania* associated with other *Beltrania*-like genera. A synoptic table of *Hemibeltrania* species is provided.

Key words — asexual *Ascomycetes*, plant pathogen

Introduction

When revisiting *Beltrania* and related genera, Pirozynski (1963) introduced the genus *Hemibeltrania* (typified by *H. cinnamomi*) for a causal fungus of cinnamon leaf spot in Sierra Leone characterized by conidia described as ellipsoid to obovate and 12–16 × 10–12 μm. The phylogenetic placement of *Hemibeltrania* is unknown.

In December 2014, a survey was conducted to investigate microfungal diversity in the natural forests of Kaladi and the surrounding area within the southern Western Ghats, India. An unusual species of *Hemibeltrania* was discovered, which causes foliar blotches in *Cinnamomum malabatrum*. The present study aims to authenticate the species of *Hemibeltrania* using morphological characters and resolve the phylogenetic position of the genus using ITS and LSU sequence data.

Materials & methods

Isolates and morphology—Conidia were directly isolated from the surface of a leaf spot and observed under a Nikon stereomicroscope (Model SMZ–1500 with Digi-CAM). Single conidial cultures were established on 2% Potato Dextrose Agar (PDA) and 2% Malt Extract Agar (MEA) plates (Crous et al. 2009). For morphotaxonomic studies and photomicrographs a Carl Zeiss Image Analyzer 2 microscope was used. Conidia and conidiophores were mounted in lactic acid cotton blue and measured using AxioVision REL.4.8 software, with 30 observations per structure. Colony characteristics in culture were studied on MEA and PDA (Crous et al. 2009). Herbarium specimens were deposited in Ajrekar Mycological Herbarium (AMH 9667), and the culture was accessioned and preserved in the National Fungal Culture Collection of India (NFCCI-3695), Agharkar Research Institute, Pune, India.

DNA extraction, amplification, and phylogenetic analyses—Colonies of *H. cinnamomi* were grown on MEA plates, and genomic DNA was extracted following the rapid salt-extraction method of Aljanabi & Martinez (1997). Fragments containing the region encoding the 28S nrDNA (LSU) and ITS 1-5.8S nrDNA-ITS 2 (ITS) were amplified using primer pairs LROR (Rehner & Samuels 1994) and LR7 (Vilgalys & Hester 1990) for LSU and primer pairs ITS4 and ITS5 (White et al. 1990) for ITS. PCR conditions, sequence alignments, and phylogenetic analyses followed the protocols of Crous et al. (2006). Thirty LSU sequences of the order *Xylariales* and an outgroup of two sequences (*Trichoderma* and *Lulworthia*) were downloaded from GenBank. Twenty-seven ITS sequences of the order *Xylariales* and an outgroup of two sequences (*Trichoderma* and *Lulworthia*) were downloaded also for phylogenetic analyses. The ITS and LSU regions were analysed by the Maximum Parsimony method with bootstrap (BS) analysis using 1000 replications in MEGA6 (Molecular Evolutionary Genetics Analysis version 6.0). Sequence data were deposited in GenBank. Family placement of the sequences follows Shirouzu et al. (2010) and Hidayat et al. (2014).

Taxonomy

Hemibeltrania cinnamomi (Deighton) Piroz., Mycol. Pap. 90: 32 (1963) Figs 1, 2

Symptoms: necrotic, reddish brown to brick brown, amphigenous, irregular.

On the natural substrate: Conidiophores distinct, single or slightly branched toward the apex, erect, straight or flexuous, cylindrical, slightly sinuate, 4–8-septate, smooth, dark brown at the base, pale brown toward the apex, thin-walled, 130–250 × 3.5–6 µm, arising from a radially lobed basal cell, 10–11.5 µm diam. Conidiogenous cells mono- and polyblastic, integrated or discrete, terminal, determinate or sympodial extended, denticulate; denticles cylindrical. Conidial secession schizolytic. Conidia solitary, limoniform, broadly ellipsoidal or obovate to irregular, rounded or obtuse at the apex, sometimes attenuate, subulate or rounded at the base with a small frill at the base, acrogenous becoming acropleurogenous after several sympodial extensions, unicellular, smooth, 12–25 × 8–13 µm, hyaline, subhyaline to pale olivaceous.

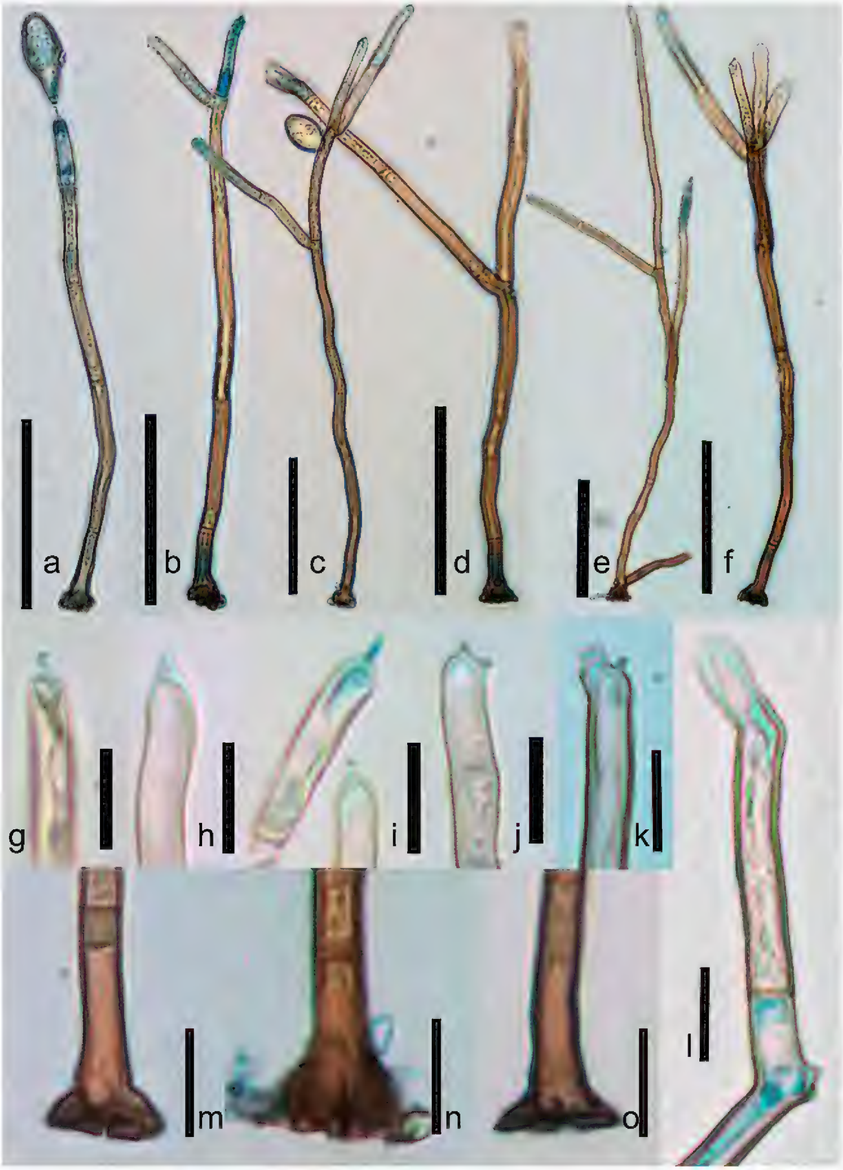

Fig. 1. *Hemibeltrania cinnamomi* (AMH 9667).
a–f: Conidiophore branching. g–l: Denticulate conidiogenous cells. m–o: Conidiophore bases.
Bars: a–f = 50 µm; g–o = 10 µm.

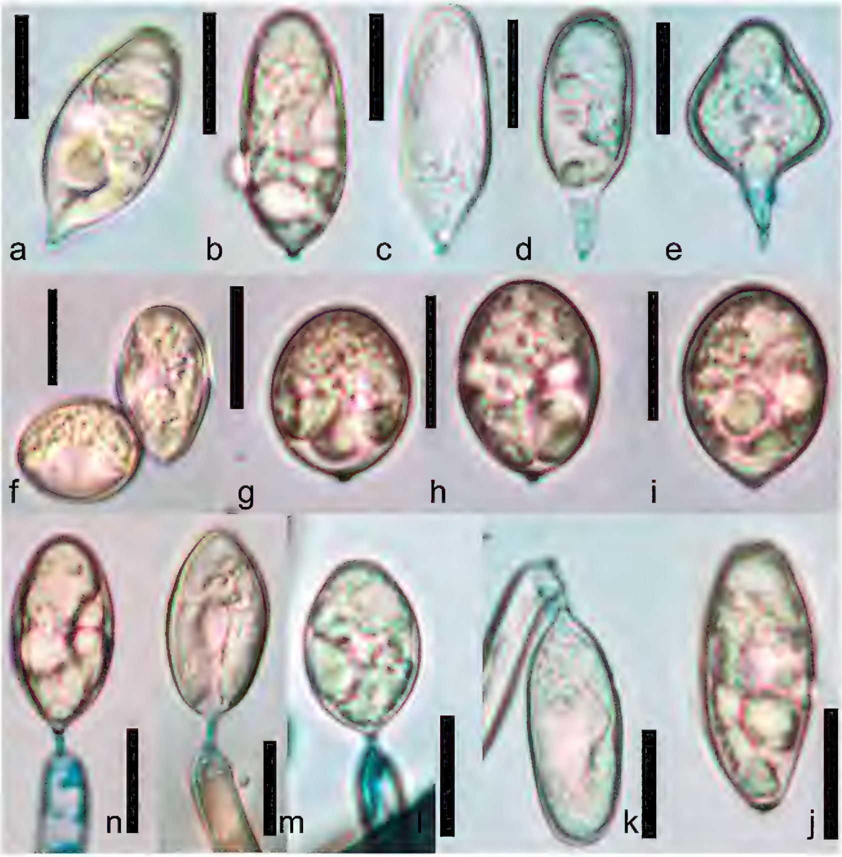

Fig. 2. *Hemibeltrania cinnamomi* (AMH 9667).
a–j: Conidia. k–n: Conidial attachments. Bars: 10 µm.

Colonies on MEA slow to moderate growing, 35–45 mm diam after 7 days, grayish or mouse gray, later turning dark gray, velutinous, margin irregular, reverse blackish to blackish blue. Conidia 12–17 × 9.5–12 µm.

Specimen examined: **INDIA. Kaladi: Western Ghats**, Kerala, 10°16′N 76°43′E, on leaves of Cinnamomum malabatrum (Burm. f.) J. Presl (*Lauraceae*) December 2014, K.C. Rajeshkumar & K. Madhusudhanan (AMH 9667; culture NFCCI 3695; Genbank KT119564, KT119565).

Phylogenetic analyses

LSU (KT119565): based on MegaBLAST search of GenBank LSU sequences, the closest hits are: *Xylaria* sp. (JQ862647.1, identities = 96%, gaps 0.2%);

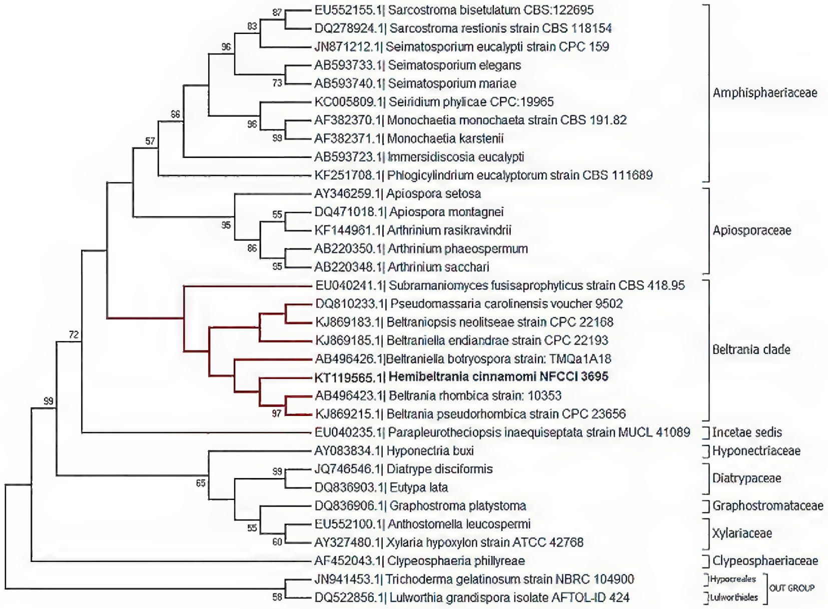
87
83
96
73
56
96
99
57
95
55
86
95
72
97
99
65
99
55
60
58
EU552155.1| Sarcostroma bisetulatum CBS:122695
DQ278924.1| Sarcostroma restionis strain CBS 118154
JN871212.1| Seimatosporium eucalypti strain CPC 159
AB593733.1| Seimatosporium elegans
AB593740.1| Seimatosporium mariae
KC005809.1| Seiridium phylicae CPC:19965
AF382370.1| Monochaetia monochaeta strain CBS 191.82
AF382371.1| Monochaetia karstenii
AB593723.1| Immersidiscosia eucalypti
KF251708.1| Phlogicylindrium eucalyptorum strain CBS 111689
AY346259.1| Apiospora setosa
DQ471018.1| Apiospora montagnei
KF144961.1| Arthrinium rasikravindrii
AB220350.1| Arthrinium phaeospermum
AB220348.1| Arthrinium sacchari
EU040241.1| Subramaniomyces fusisaprophyticus strain CBS 418.95
DQ810233.1| Pseudomassaria carolinensis voucher 9502
KJ869183.1| Beltraniopsis neolitseae strain CPC 22168
KJ869185.1| Beltraniella endiandrae strain CPC 22193
AB496426.1|Beltraniella botryospora strain: TMQa1A18
KT119565.1| **Hemibeltrania cinnamomi NFCCI 3695**
AB496423.1| Beltrania rhombica strain: 10353
KJ869215.1| Beltrania pseudorhombica strain CPC 23656
EU040235.1| Parapleurotheciopsis inaequiseptata strain MUCL 41089
AY083834.1| Hyponectria buxi
JQ746546.1| Diatrype disciformis
DQ836903.1| Eutypa lata
DQ836906.1| Graphostroma platystoma
EU552100.1| Anthostomella leucospermi
AY327480.1| Xylaria hypoxylon strain ATCC 42768
AF452043.1| Clypeosphaeria phillyreae
JN941453.1| Trichoderma gelatinosum strain NBRC 104900
DQ522856.1| Lulworthia grandispora isolate AFTOL-ID 424
Amphisphaeriaceae
Apiosporaceae
Beltrania clade
Incetae sedis
Hyponectriaceae
Diatrypaceae
Graphostromataceae
Xylariaceae
Clypeosphaeriaceae
Hypocreales
Lulworthiales
OUT GROUP

Fig. 3. LSU phylogenetic tree generated from Maximum Parsimony (MP) analyses of the aligned sequences of *Hemibeltrania cinnamomi* and allied taxa of the order *Xylariales*, with *Trichoderma* and *Lulworthia* as outgroup. Percentage bootstrap support (≥50%) is shown on the branches. The MP tree was obtained using the Subtree-Pruning-Regrafting algorithm with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). The bootstrap test was conducted with 1000 replicates.

Xylaria sp. (JQ862650.1, identities = 96%, gaps = 0.2%); and *Xylaria badia* (JQ862643.1, identities = 96%, gaps = 0.2%).

ITS (KT119564): A MegaBLAST of the ITS sequence showed that the closest hits are: *Hemibeltrania* sp. CL12WA (JQ621881.1, identities = 99%, gaps 0.4%); *Beltraniella endiandrae* CPC 22193 (KJ869128.1, identities = 93%, gaps = 3%); and *Beltrania pseudorhombica* CPC 23656 (KJ869158.1, identities = 92%, gaps = 4%).

The ITS and LSU phylogenetic analyses supported the genus *Hemibeltrania* as closely related to *Beltrania*, *Beltraniopsis*, *Beltraniella*, and *Subramaniomyces* and forming a unique monophyletic lineage in the *Xylariales* and closely allied to *Amphisphaeriaceae* and/or *Apiosporaceae* (Figs 3, 4).

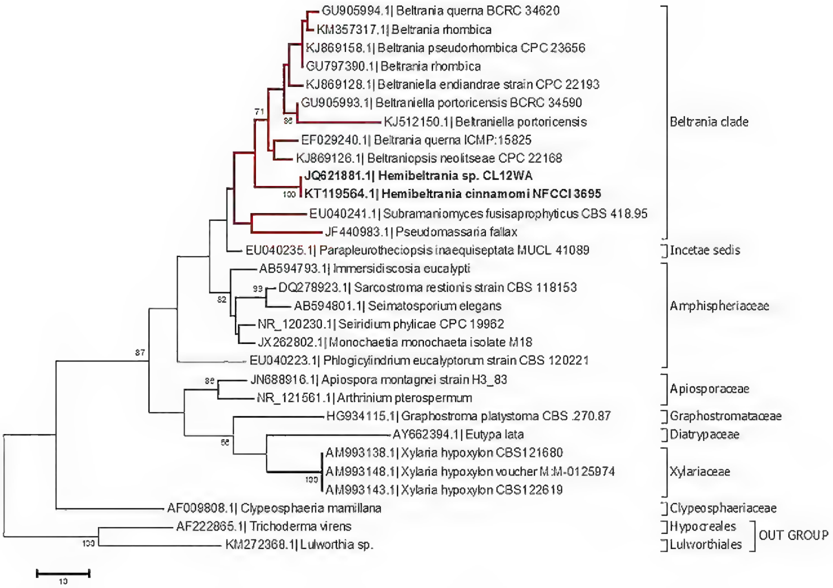
GU905994.1| Beltrania querna BCRC 34620
KM357317.1| Beltrania rhombica
KJ869158.1| Beltrania pseudorhombica CPC 23656
GU797390.1| Beltrania rhombica
KJ869128.1| Beltraniella endiandrae strain CPC 22193
GU905993.1| Beltraniella portoricensis BCRC 34590
KJ512150.1| Beltraniella portoricensis
EF029240.1| Beltrania querna ICMP:15825
KJ869126.1| Beltraniopsis neolitseae CPC 22168
JQ621881.1| Hemibeltrania sp. CL12WA
KT119564.1| Hemibeltrania cinnamomi NFCCI 3695
EU040241.1| Subramaniomyces fusisaprophyticus CBS 418.95
JF440983.1| Pseudomassaria fallax
EU040235.1| Parapleurotheciopsis inaequiseptata MUCL 41089
AB594793.1| Immersidiscosia eucalypti
DQ278923.1| Sarcostroma restionis strain CBS 118153
AB594801.1| Seimatosporium elegans
NR_120230.1| Seiridium phylicae CPC 19962
JX262802.1| Monochaetia monochaeta isolate M18
EU040223.1| Phlogicylindrium eucalyptorum strain CBS 120221
JN688916.1| Apiospora montagnei strain H3_83
NR_121561.1| Arthrinium pterospermum
HG934115.1| Graphostroma platystoma CBS 270.87
AY662394.1| Eutypa lata
AM993138.1| Xylaria hypoxylon CBS121680
AM993148.1| Xylaria hypoxylon voucher M:M-0125974
AM993143.1| Xylaria hypoxylon CBS122619
AF009808.1| Clypeosphaeria mamillana
AF222865.1| Trichoderma virens
KM272368.1| Lulworthia sp.
71
88
100
93
82
87
86
56
100
100
Beltrania clade
Incetae sedis
Amphisphariaceae
Apiosporaceae
Graphostromataceae
Diatrypaceae
Xylariaceae
Clypeosphaeriaceae
Hypocreales
Lulworthiales
OUT GROUP
10

Fig. 4. ITS phylogenetic tree generated from Maximum Parsimony (MP) analyses of the aligned sequences of *Hemibeltrania cinnamomi* and allied taxa, with *Trichoderma* and *Lulworthia* as outgroup. Percentage bootstrap support (≥50%) is shown on the branches. The MP tree was obtained using the Subtree-Pruning-Regrafting algorithm with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). The bootstrap test has 1000 replicates.

Discussion

Hemibeltrania comprises 12 accepted species (MycoBank 2015; Table 1). Taxonomic study of the present collection revealed that in *H. cinnamomi,* conidial shapes and sizes are more variable than the original description: conidia are longer than 10 µm, while conidial shapes range from limoniform, obovate, and broadly ellipsoid to irregular. The symptomatology and other taxonomic characters, however, are identical to the type description given by Pirozynski (1963). Considering the variable conidial shape and size observed in AMH 9667, we propose a broad species concept for *H. cinnamomi* that might be expanded in the future based on re-examination and phylogenetic analysis of *H. nectandrae*, which shows some affinity with *H. cinnamomi.*

Earlier studies have shown that *Beltrania* and related genera (*Beltraniella*, *Beltraniopsis*, *Subramaniomyces*) are phylogenetically allied with *Amphisphaeriacaeae*, *Xylariales* (Shirouzu et al. 2010, Hidayat et al. 2014). Shirouzu et al. (2010) also noted that *Beltraniella* anamorphs and

TABLE 1. Comparative morphology of accepted *Hemibeltrania* species

SPECIES	CONIDIA		CONIDIOPHORE SIZE (µm)
	SHAPE	SIZE (µm)	
H. cinnamomi	Obovoid/broadly ellipsoid/limoniform	12–25 × 8–13	130–250 × 3.5–6
H. convallariae	Ovoid	14–24 × 10–15	≤2000 × 13–23
H. cymbiformis	Cymbiform	21–24 × 3–4.5	≤140 × 2.2–4.5
H. decorosa	Fusiform	17–22 × 3.5	≤250 × 4–6.5
H. echinulata	Ovoid	12–14 × 5.5–7	140–300 × 3.5–5
H. laxa	Obclavate/navicular	13–17 × 2–3	50–350 × 3–7
H. malaysiana	Ellipsoid/navicular	13–20 × 3–5	≤400 × 4–7
H. mitrata	Mitriform	14–17 × 4.5–5.5	35–85 × 3–4.5
H. nectandrae	Limoniform	15–23 × 10–13	≤250 × 4–6
H. ovalispora	Obovoid	12–14 × 7	≥750 × 4–6
H. saikawae	Clavate/cylindrical	14–19 × 2.5–3	10–155 × 2–3
H. urbanodendri	Ovoid/obovoid/ biconic/limoniform	12–25 × 6–10	55–106 × 4–6

Pseudomassaria teleomorphs are phylogenetically linked. Our phylogenetic analysis of *H. cinnamomi* does support *Hemibeltrania* within the *Xylariales*. However, the family in which it should be placed remains ambiguous.

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